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Water-stress-induced changes in the abscisic acid content of guard cells and other cells of *Vicia faba* L. leaves as determined by enzyme-amplified immunoassay

(stomata/gas exchange/plant growth regulator/transpiration/photosynthesis)

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ABSTRACT **A highly sensitive, solid-phase, enzyme-amplified immunoassay for the plant growth regulator (+)-abscisic acid (ABA) was developed. The assay sensitivity (0.2–10 fmol) was sufficient for analyzing free ABA in homogeneous tissue samples dissected from *Vicia faba* L. leaves. Eight hours after detached leaves had been desiccated to 10% loss of fresh weight, the bulk leaf ABA content increased from ≤0.2 to 6.2 ng·(mg dry weight)$^{-1}$. Epidermal tissue, spongy parenchyma cells, and palisade parenchyma cells from this water-stressed leaf had the following ABA contents, respectively: 4.8, 9.4, and 9.0 ng·(mg dry weight)$^{-1}$. Guard cells, which respond to exogenous ABA by losing solutes and volume, were also assayed. When they were dissected from control (fully hydrated) leaves, their ABA content was ≈0.7 fg·(cell pair)$^{-1}$ [≈0.2 ng·(mg dry weight)$^{-1}$]. In contrast, the ABA content of guard cells of water-stressed leaves was ≈17.7 fg·(cell pair)$^{-1}$. These results indicate that ABA accumulation in a highly stressed *V. faba* leaflet is generalized; guard cells contain only 0.15% of bulk leaf ABA. The time course for loss of ABA from guard cells of a floating epidermal peel was studied. There was little loss within 30 min, but after 4 hr, the ABA content was only 17% of the original value. These results indicate that the bulk of guard cell ABA is not readily diffusible (i.e., probably not apoplastic). The results also indicate that common laboratory procedures result in lowered guard cell ABA content.**

Water vapor is lost from a leaf through the same epidermal pores, the stomata, that admit CO_2 for photosynthetic reduction. The rate of gas exchange is regulated by aperture size, which varies as a result of volume changes in the pair of guard cells that surrounds the pore. These volume changes occur in response to environmental stimuli (e.g., light and humidity), and internal stimuli (like plant water deficit or CO_2 concentration). Thus, resistance to water-vapor loss and CO_2 uptake are integrated with ambient and physiological conditions. When a plant is water-stressed, the stomata close. This response, as indicated, reduces water-vapor loss by transpiration; therefore, the plant is somewhat protected from the deleterious effects of a transient stress condition, such as drought.

Abscisic acid (ABA), an endogenous growth regulator, is postulated to mediate stress-induced stomatal closure. This hypothesis is supported by the observations (for reviews, see refs. 1–4) that (*i*) ABA synthesis is stimulated by water-stressing a leaf, (*ii*) ABA concentration is much elevated in a water-stressed leaf, (*iii*) stomatal aperture size is smaller in a water-stressed leaf and usually negatively correlated with leaf ABA concentration, and (*iv*) exogenous ABA induces stomatal closure. The hypothesis is further supported by identification of wilty mutants that do not respond to water stress by stomatal closure (5). These mutants have impaired facility for ABA synthesis. Although such correlative data are abundant, inconsistencies in the relationship between ABA content and stomatal behavior have also been reported. For example, water-stress-induced stomatal closure often occurs before increases in "bulk leaf" ABA content (e.g., refs. 6–9). Such observations are the basis for speculation that redistribution of ABA within the leaf is sufficient for stomatal effects (10, 11). Presumably, the migration of ABA from storage sites to the guard cell plasmalemma (12, 13) would precede detectable increase in ABA synthesis. There is no experimental basis for this hypothesis, however.

Mesophyll cells are the CO_2 sink, and until recently it was believed that these cells, as the source of ABA, modulated stomatal aperture size. Two lines of evidence supported this belief: (*i*) the bulk of ABA in well-watered leaves is in mesophyll chloroplasts (14), and (*ii*) epidermal tissue—it was reported (7, 14)—is unable to synthesize ABA. However, recent evidence indicates that guard cells synthesize ABA (15, 16) and may metabolize it (17). Indeed, analyses of flux rates indicate that the guard cell ABA concentration exceeds considerably that of whole leaf (18). Together, these recent reports (15–18) imply that guard cells themselves might release the ABA to their immediate environment and, thus, stimulate closure of the pore. However, there have been few attempts to measure the ABA content of guard cells or changes in ABA content that occur when water stress is imposed. Here, we describe methods for direct measurement of ABA in guard cells by an enzyme-amplified immunoassay. Using this assay, we have determined directly the *in situ* ABA content of guard cells from control and water-stressed leaves. For comparison, we report also ABA content of other cells.

MATERIALS AND METHODS

Plant Material. *Vicia faba* L. cv. Longpod plants (seeds from W. Atlee Burpee, Warminster, PA) were grown in a vermiculite/peat mixture, 1:1 (vol/vol), in a growth cabinet with the following conditions: light intensity, 400–700 nm (600 $\mu E \cdot m^{-2} \cdot s^{-1}$); day/night temperatures, 25°C/20°C; relative humidity, 60%; photoperiod, 15 hr. Young, fully expanded, bifoliate leaves (one leaf per plant) on 3- to 5-week-old plants were used in all experiments. One of two sister leaflets (control sample) was detached, quickly frozen in a liquid N_2 slurry (approximately −215°C), and freeze-dried at −35°C and 10 μmHg for 48 hr. The other leaflet (water-

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Abbreviations: ABA, abscisic acid; INT, *p*-iodonitrotetrazolium violet.

stressed sample) was detached, dehydrated to a 10% fresh weight loss, and stored in a sealed plastic bag for 8 hr in darkness at 23°C (16). Then this leaflet was also frozen and dried as described above. The dried tissue was stored under vacuum at −20°C until used. Guard cell pairs (Fig. 1) and other cells were dissected out and weighed as described elsewhere (19, 20), where further details are given.

In a separate experiment, epidermal peels (21) taken from the abaxial surfaces of either control or water-stressed leaflets were floated (cuticular side up) on ice-cold water. At indicated times (see Fig. 4), samples were frozen and then dried as above.

Chemicals. Alkaline phosphatase (EC 3.1.3.1) was from Boehringer Mannheim. Dihydrolipoamide reductase (NAD^+) ("diaphorase," type II-L; EC 1.6.4.3) and most other biochemicals were from Sigma.

Glassware. Glassware was treated for 10 min with 5% (vol/vol) dimethyldichlorosilane in hexane; successively rinsed with hexane, methanol, and hexane; and dried at 100°C for 24 hr (as recommended by R. Morris, personal communication).

Extraction Procedure. ABA was extracted from guard cells in a 50-nl droplet of 80% aqueous (vol/vol) methanol (amended to include 10 mg of 2,6-di-*t*-butyl-*p*-cresol per liter) under oil (see refs. 19 and 20) for 24 hr at 4°C in the dark. The methanolic extracts were diluted with Tris-buffered saline (TBS; 50 mM Tris, pH 7.8/1 mM $MgCl_2$/10 mM NaCl).

ABA was extracted from other tissue samples (25–50 μg dry weight) with 80% aqueous (vol/vol) and then 100% methanol (2 μg of tissue dry weight per μl of extract) for 24 and 6 hr, respectively, at 4°C in darkness in 6 × 10 mm borosilicate glass tubes. The extracts were combined and dried under N_2. The resulting residue was dissolved in a small volume of methanol and diluted with TBS.

In some cases, abscisyl β-D-glucopyranoside was added to parallel extracts to determine potential overestimation of ABA resulting from methanolysis of ABA conjugates during extraction.

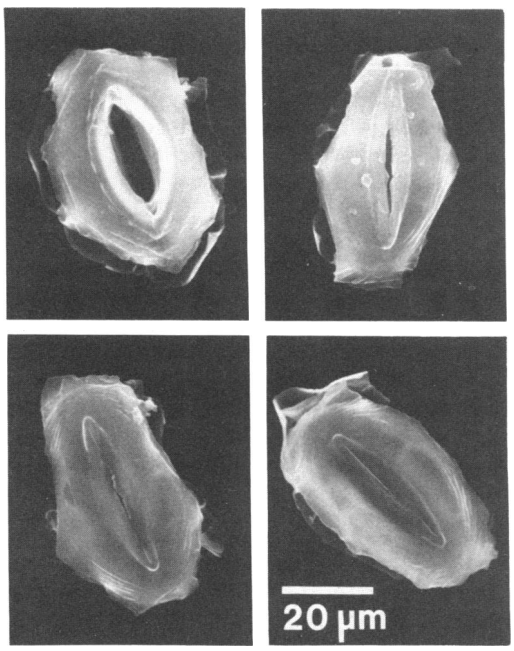

FIG. 1. Scanning electron micrographs of guard-cell pairs. Guard-cell pairs were excised, as in the experimental protocol, from freeze-dried *V. faba* leaves. Then, the cells were transferred to a glass coverslip, coated with carbon, and directly examined for contamination.

Synthesis of Alkaline Phosphatase-Labeled ABA. Racemic ABA was conjugated to alkaline phosphatase as described by Mertens (22), except that the 4′-*p*-aminobenzoyl hydrazon derivative of ABA was purified by repeated TLC (four times on 250-μm silica GF with a solvent system of toluene/ethyl acetate/acetic acid, 50:30:4 (vol/vol); $R_f \approx 0.05$). The enzyme conjugate was stored in 50% (vol/vol) glycerol in TBS at −20°C with no detectable loss in either enzymic activity or immunoreactivity over a 1-year period.

Enzyme-Amplified Immunoassay Procedure. *General aspects.* Micromethods of sample preparation and extraction were combined with a highly sensitive (2×10^{-16} mol of ABA per assay), low-volume, solid-phase, enzyme-amplified immunoassay for ABA. The assay can be divided into three parts: (*i*) a competitive binding step in which alkaline phosphatase-labeled ABA competes with a sample of ABA for immobilized, high-affinity ($K_a = 10^9\ M^{-1}$), monoclonal, anti-(+)-ABA binding sites [previously (23), this antibody was shown by HPLC to be reactive with only (+)-ABA]; (*ii*) a specific reaction step in which antibody-bound alkaline phosphatase hydrolyzes NADP to NAD; and (*iii*) an amplification step in which the NAD (≈1–10 pmol) is enzymically cycled, resulting in the accumulation of reduced *p*-iodonitrotetrazolium violet (INT) (24, 25).

Enzyme-Amplified Immunoassay Procedure. *Specific aspects.* Wells of Terasaki-type tissue-culture plates (10-μl capacity per well; Nunclon Delta Microwell Plates, Nunc, Denmark) were filled with 1 μl of a solution (250 $\mu g \cdot ml^{-1}$) of affinity-purified, polyclonal, rabbit anti-mouse IgG in 50 mM carbonate buffer (pH 9.6) and incubated at 4°C for 24 hr. The coating solution was decanted, and the plates were rinsed four times with TBS. To each well was added 1 μl of TBS containing monoclonal, anti-(+)-ABA antibody cell line 15-I-C5, antibody subclass IgG_1, at 50 $\mu g \cdot ml^{-1}$ (23). The plates were incubated for an additional 24 hr at 4°C. The coating solution was decanted, and the plates were rinsed four times with TBS.

To each coated well was added 0.6 μl of ABA standard or the unknown sample diluted in TBS. After incubation for 1 hr at 4°C, 0.4 μl of diluted alkaline phosphatase-labeled ABA (1:750 dilution was in 0.1% gelatin/TBS) was added, and the plate was incubated for an additional 3 hr. The plate contents were discarded, and the plate was rinsed four times with TBS.

Bound enzyme activity was determined by addition to each well of 2 μl of 200 μM NADP in 50 mM diethanolamine (pH 9.5) containing 1 mM $MgCl_2$. The plate was incubated at 37°C for 2 hr. (This long incubation reduced to insignificance minor differences in incubation time for each well that were incurred during aliquoting.) The contents of each well were then transferred to corresponding wells of a 96-well, flat-bottom, immunoassay plate (400-μl capacity per well; Immuno Plate I, Nunc, Denmark). Alkaline phosphatase-dependent NAD production was determined by enzymic cycling (24, 25). Briefly, 200 μl of cycling reagent [50 mM Tris (pH 7.5) containing 300 mM ethanol, 1 mM INT, alcohol dehydrogenase at 40 $\mu g \cdot ml^{-1}$, and diaphorase at 100 $\mu g \cdot ml^{-1}$ (stored as a 3 $mg \cdot ml^{-1}$ stock solution in 200 mM Tris, pH 7.5/300 mM KCl/0.5 mM FMN/0.025% bovine serum albumin] was added to each well of the large-volume plate. The plate was incubated at 23°C, and the colored reaction product was allowed to accumulate for 15 min, over which period the indicator reaction was linear. This amplification step was terminated by addition of 50 μl of 0.3 M HCl to each well. The absorbance at 490 nm was measured.

Additional comments. The enzyme tracer dilution was arbitrarily selected so that the end-point absorbance in the absence of standard or sample equaled 1.0. Unspecific binding of tracer was determined with an excess (100 fmol)

of ABA and equaled <5% of the blank (no added ABA) under these conditions.

RESULTS

Assay Characteristics. Standard curves in triplicate were routinely run on all plates along with unknown samples. In Fig. 2 a composite standard curve (six separate assays ± SD) and a linear logit–log transformation of the standard data are presented. The measuring range of the assay (defined on the range of 10% to 90% tracer displacement) was from 200 amol to 10 fmol (≈50–2500 fg) of ABA. The slope of the logit–log transformation, an indication of assay precision, equaled −1.23. The assay ED_{50}, or the dosage that displaced 50% of the tracer, equaled 1.36 fmol of ABA. Coefficients of variation for triplicate analyses of standards throughout the linear range of the curve were typically between 5% and 10%. The variation in unknown samples did not exceed this value.

Sample Preparation and Assay. Methanolic leaf-tissue extracts were dried under N_2, dissolved in a small volume of methanol, and diluted with TBS. In contrast, methanolic guard cell extracts were made under oil and were directly diluted with TBS (1 μl of TBS per 50-nl extraction droplet). In no case did the final methanol concentration exceed 5% (vol/vol), whereas the assay was not affected by 8%, the highest concentration tested.

The ratio of tissue dry mass to extractant volume (g/liter) was 2:1 in the case of leaf tissues extracted by conventional techniques but ranged from 1:1 to as high as 10:1 for guard cell samples. However, based on the consistency of results obtained with different numbers of cells per droplet (as will be seen in Table 2), this difference apparently had no effect on extraction efficiency. As added proof, equivalent results were obtained when palisade tissue was extracted either under oil as excised single cells or by conventional techniques using larger tissue masses (data not shown).

All assays were conducted on crude extracts without additional purification. Although the use of unpurified plant extracts has been validated by RIA for the antibody (23) we used, the absence of interfering compounds was routinely confirmed by internal standardization and by demonstration of parallelism of extract dilution curves with the standard curve (see ref. 26). With internal standardization, a constant volume of extract was assayed with and without the addition of known amounts of ABA standard. The amount of ABA determined by enzyme immunoassay was then plotted as a function of the amount of ABA added. In the absence of interfering compounds, the plot yields a straight line with a slope of 1 and a *y* intercept equal to the amount of ABA contained in the extract. The results of one experiment are presented in Fig. 3. ABA content of this extract was 8.7 ng·(mg dry weight)$^{-1}$ of original tissue. Dilution analysis of this extract over a 4-fold range was in close agreement with this result [8.4 ± 0.4 (SD) ng·(mg dry weight)$^{-1}$].

Because *V. faba* tissues, including the guard cells, possibly contain substantial amounts of conjugated ABA (e.g., abscisyl β-D-glucopyranoside; see ref. 17), the potential overestimation of ABA due to methanolysis of ABA conjugates (27) was tested by addition of abscisyl β-D-glucopyranoside standards to parallel extracts. No increase in ABA content was observed when tissue samples were spiked with methanolic solutions of this substance and extracted as described (data not shown). This result is consistent with the previously reported nonimmunoreactivity of both the glucosyl and methyl esters of ABA (23).

ABA Levels in Various Tissues of Control and Water-Stressed Leaves. Eight hours after detached leaves had been desiccated to a 10% loss of fresh weight, the bulk leaf ABA content had increased about 30-fold [0.2 vs. 6.2 ng·(mg dry weight)$^{-1}$; Table 1]. The highest ABA content found in water-stressed leaf tissue was observed in the mesophyll, and the lowest was observed in the epidermal tissue [9.4 and 9.0 ng·(mg dry weight)$^{-1}$ for the spongy and palisade parenchyma, respectively, vs. 4.4 ng·(mg dry weight)$^{-1}$ for the epidermis; Table 1]. [The value for epidermal tissue is little affected by guard cells in the samples because, on mass basis, guard cells are only about 5% of the epidermal tissue on a dry weight basis; see the next paragraph.]

An increase in the ABA content of guard cells was observed in the water-stressed leaf [0.7 vs. 17.7 fg·(guard cell pair)$^{-1}$, respectively, for control and water-stressed tissue; Table 2]. Assuming a dry weight of 4.8 ± 0.8 ng·(guard cell pair)$^{-1}$ (present study, $\bar{x}$ ± SD; n = 20), the ABA contents of guard cells were 0.15 and 3.7 ng·(mg dry weight)$^{-1}$, respectively, for control and water-stressed leaf tissue.

ABA Levels in Guard Cells of Floated Epidermal Strips. When epidermal strips were prepared from water-stressed leaves and floated on ice-cold water, the ABA content of the guard cells decreased with time (Fig. 4). After 0.5, 1, and 4 hr, the guard cell ABA content had declined to approximately 87%, 75%, and 17%, respectively, of its original

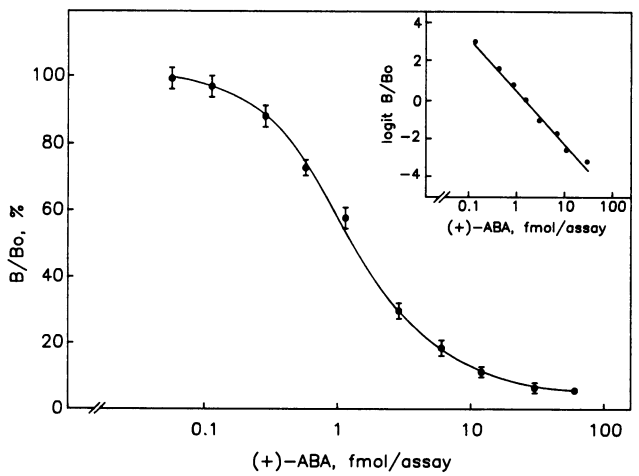

FIG. 2. Enzyme immunoassay curve for (+)-ABA ($\bar{x}$ ± SD; n = 6 separate assays). B/Bo is the relative binding (%) of tracer in the presence (B) or absence (Bo) of standards. (*Inset*) Logit–log plot. Logit B/Bo = ln [(B/Bo)/(100 − B/Bo)].

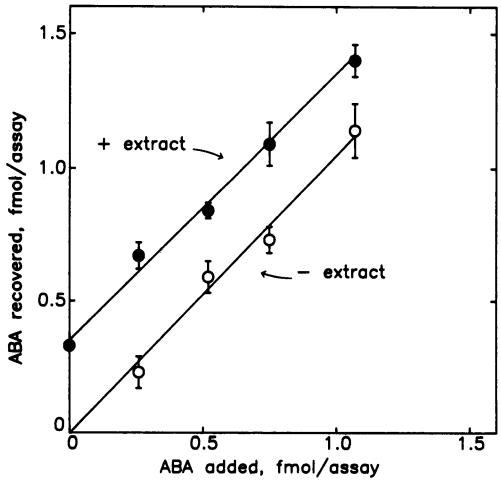

FIG. 3. Internal standard curve at one level of sample dilution to demonstrate the absence of interfering compounds; the tissue analyzed was water-stressed whole-leaf tissue. Data are presented as $\bar{x}$ ± SD (n = 3).

Table 1. ABA in tissues of control and water-stressed *V. faba* L. leaflets

Tissue	ABA, ng·(mg dry weight)$^{-1}$	
	Water-stressed*	Control
Whole leaf	6.2 ± 0.3	≤0.2
Epidermis†	4.8 ± 0.4	—
Spongy parenchyma	9.4 ± 0.6	—
Palisade parenchyma	9.0 ± 0.8	—

*A pair of sister leaflets was detached from well-watered plants. The control leaflet was quenched immediately. The other leaflet was water-stressed by dehydration to 90% of its original fresh weight and incubation in darkness for 8 hr before quenching. Data are expressed as $\bar{x}$ ± SD and were obtained from leaflet 1, Table 2.
†The uniserate abaxial epidermis comprising several types of cells.

value. Although the ABA content of the medium was not assayed, the ABA decline was probably a result of ABA loss, because little metabolism would be expected at this temperature.

DISCUSSION

Our results indicate that the enzyme-amplified immunoassay provides a sensitive method for measuring ABA in a range from 200 amol to 10 fmol. This range is 100 times more sensitive than other published immunoassays for ABA (see ref. 28); it is also considerably more sensitive than traditional physicochemical methods for ABA (e.g., ref. 29), which cannot be applied to single-cell investigations. In combination with micromethods of sample preparation (19, 20), the enzyme-amplified immunoassay is useful for measuring ABA in unpurified extracts of water-stressed tissue samples that are as small as 50 ng dry weight (e.g., fewer than four spongy parenchyma cells). Because equivalent antibodies for other plant growth regulators are becoming available, these general techniques should be applicable for many types of studies where high morphological resolution is required. However, prior to the application of these techniques to the analyses of other plant growth regulators, careful consideration of antibody specificity and the demonstrated absence of potentially interferring compounds, as done in the present study, is essential.

Table 2. ABA in guard cells from control and water-stressed *V. faba* L. leaflets

Guard-cell pairs per assay	ABA, fg·(guard-cell pair)$^{-1}$	
	Water-stressed*	Control
Leaf 1		
100	—	0.8
50	21.5	≤1.0†
20	17.2	—
10	18.8	—
Averages	19.2 ± 2.2 (SD)	0.8
Leaf 2		
100	—	0.7
50	14.9	≤1.0†
20	15.2	—
10	16.3	—
Averages	15.5 ± 0.7 (SD)	0.7
Leaf 3		
100	—	0.7
50	18.5	≤1.0†
20	17.2	—
10	19.5	—
Averages	18.5 ± 1.2 (SD)	0.7

*See Table 1 for stress treatment.
†Two experiments.

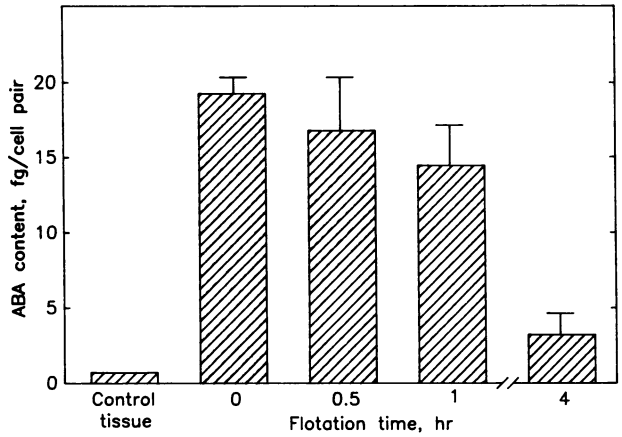

FIG. 4. The decrease in guard-cell ABA content in epidermal peels that were prepared from water-stressed leaves and then floated on ice-cold water. Data are presented as $\bar{x}$ ± SD (n = 3).

In the present study, we measured the water-stress-induced changes in the ABA content of various leaf tissues, including guard cells. Our results show that guard cells of unstressed leaves have a low but detectable ABA content. Our results also demonstrate unequivocally the *in situ* accumulation of ABA in guard cells of water-stressed leaves (Table 2). Although the source of guard-cell ABA is unknown, the accumulation appears to be a generalized phenomenon, because similar changes in ABA content were observed in all leaf tissues (Tables 1 and 2). If expressed on a cell basis, the ABA content of guard cells from water-stressed leaves was less than that observed in the mesophyll (compare 37.5 amol per cell with 395 and 490 amol per cell, values for palisade parenchyma and spongy parenchyma cells, respectively; palisade and spongy parenchyma cell masses are from ref. 30). However, if differences in cell volumes are taken into account (see ref. 31), the ABA concentrations in the different water-stressed cells are similar and range from 7 to 13 μM (ignoring cellular compartmentation).

Our values for ABA levels in water-stressed guard cells are 9-fold higher than those of Weiler *et al.* (15), who made the measurements on guard cell protoplasts; they are 8-fold higher than the values reported by Cornish and Zeevaart (16), who made measurements on sonicated epidermal peels. Raschke (32) has discussed the "peculiar situation" of guard cells in epidermal strips with regard to solute loss. Cornish and Zeevaart (16) make explicit their concern that the "levels . . . may well underestimate the actual" because of documented ABA efflux from leaf slices and roots. We show that their concern is valid (Fig. 4). The simple result is that ABA content of guard cells of epidermal strips declines when the strips are floated on water. This observation calls into question quantitation of ABA in tissue that has been treated in any way. Another important conclusion is that guard cell ABA does not decline immediately when epidermal strips are floated, as one would expect if it were apoplastic (see ref. 33). Indeed, the ABA content of floated epidermal strips had hardly decreased after 0.5 hr. This result indicates that the bulk of the ABA in the guard cells is either located intracellularly or, if located in the apoplast, is not readily diffusable.

In summary, water-stressed *V. faba* L. leaves accumulated ABA. Large changes in ABA content were observed in all leaf tissues, including the guard cells. The concentration of ABA found in water-stressed guard cells was in the "physiological range" for modulation of stomatal aperture (e.g., refs. 34 and 35). Although the relevance of guard cell ABA accumulation to stomatal response to water stress

remains unclear, our results emphasize the conclusion of Raschke (3) that it should not be surprising that attempts to correlate bulk leaf ABA content with stomatal closure have often resulted in contradictory results. Indeed, our results indicate that the ABA content of guard cells (including the apoplastic space) is only 0.15% of the leaf ABA. Therefore, changes in ABA content of the guard cells or their immediate environment will go undetected if only bulk leaf ABA content is measured. Resolution of the source of stomatal ABA and the relevance of such accumulation to stomatal function will depend on studies of the kinetics of ABA accumulation in the guard cells themselves when water stress is imposed.

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